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# Kongeriget Danmark

**PRIORITY DOCUMENT**

Patent application No.: 1044/93

Date of filing: 17 Sep 1993

Applicant: Novo Nordisk A/S, Novo Allé, 2880 Bagsværd, DK

The attached photocopy is a true copy of the following document:

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TAASTRUP 07 Sep 1994

Suzanne Schmitt
Kontorfuldmægtig

# NOVEL PEPTIDES

## FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

## BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the requirement related to meals.

Protracted insulin compositions are well known in the art. Thus one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus in order to secure an accurate dosing the insulin particles must be suspended homogeneously by gentle shaking before a defined volume is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic it has now turned out that protamines can be immuno-

genic in man and that their use for medical purposes may lead to formation of antibodies (Samuel, T. et al., Studies on the immunogenecity of protamines in humans and experimental animals by means of a micro-complement fixation test. Clin. Exp. Immunol. 33 (1978)252-260).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz, A.B. et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25 (1983)322-324). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback with these solutions is that the particle size distribution of the precipitate formed in the tissue on injection and thus the timing of the medication depends on the blood flow at the injection site and other parameters in a somewhat unpredictable manner. A further drawback is that the solid particles of the insulin may act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction these complexes is only intermediate and the bioavailability is low.

By "insulin derivative" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulphide bridges between $Cys^{A7}$ and $Cys^{B7}$ and between $Cys^{A20}$ and $Cys^{B19}$ and an internal disulphide bridge between $Cys^{A6}$ and $Cys^{A11}$ and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

**SUMMARY OF THE INVENTION**

Surprisingly, it has turned out that certain human insulin derivatives wherein the $\epsilon$-amino group of $Lys^{B29}$ has a lipohilic substituent have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg, and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg, and Cys; $Phe^{B1}$ may be deleted; the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 $Zn^{2+}$ ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and $Phe^{B1}$ is not deleted, then 2-4 $Zn^{2+}$ ions are bound to each hexamer of the insulin derivative.

In one preferred embodiment the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg, and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg, and Cys, with the proviso that if the B30 amino acid residue is Ala or Thr, then at least one of the residues A21 and B3 is different from Asn; $Phe^{B1}$ may be deleted; and the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which comprises at least 6 carbon atoms.

In another preferred embodiment the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg, and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg, and Cys; $Phe^{B1}$ may be deleted; the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 $Zn^{2+}$ ions are bound to each insulin hexamer.

In another preferred embodiment the invention relates to a human insulin derivative in which the B30 amino acid residue is deleted.

In another preferred embodiment the invention relates to a human insulin derivative in which the B30 amino acid residue is Asp.

In another preferred embodiment the invention relates to a human insulin derivative in which the B30 amino acid residue is Glu.

In another preferred embodiment the invention relates to a human insulin derivative in which the B30 amino acid residue is Thr.

In another preferred embodiment the invention relates to a human insulin derivative in which the A21 amino acid residue is Ala.

In another preferred embodiment the invention relates to a human insulin derivative in which the A21 amino acid residue is Gln.

In another preferred embodiment the invention relates to a human insulin derivative in which the A21 amino acid residue is Gly.

In another preferred embodiment the invention relates to a human insulin derivative in which the A21 amino acid residue is Ser.

In another preferred embodiment the invention relates to a human insulin derivative in which the B3 amino acid residue is Asp.

In another preferred embodiment the invention relates to a human insulin derivative in which the B3 amino acid residue is Gln.

In another preferred embodiment the invention relates to a human insulin derivative in which the B3 amino acid residue is Thr.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an acyl group corresponding to a carboxylic acid having at least 6 carbon atoms.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an acyl group, branched

or unbranched, which corresponds to a carboxylic acid having a chain of carbon atoms 8 to 24 atoms long.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an acyl group corresponding to a fatty acid having at least 6 carbon atoms.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an acyl group corresponding to a linear, saturated carboxylic acid having from 8 to 12 carbon atoms.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an oligo oxyethylene group comprising up to 10, preferably up to 5, oyxethylene units.

In another preferred embodiment the invention relates to a human insulin derivative in which the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which is an oligo oxypropylene group comprising up to 10, preferably up to 5, oxypropylene units.

In another preferred embodiment the invention relates to a human insulin derivative in which each insulin hexamer binds 2 $Zn^{2+}$ ions.

In another preferred embodiment the invention relates to a human insulin derivative in which each insulin hexamer binds 3 $Zn^{2+}$ ions.

In another preferred embodiment the invention relates to a human insulin derivative in which each insulin hexamer binds 4 $Zn^{2+}$ ions.

In another preferred embodiment the invention relates to the use of a human insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of a human insulin derivative according to the invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment the invention relates to a pharmaceutical composition comprising a human insulin derivative according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment the invention relates to a protracted pharmaceutical composition comprising a human insulin derivative according to the invention.

In another preferred embodiment the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of a human insulin derivative according to the invention.

In another preferred embodiment the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred human insulin derivatives according to the present invention in which no $Zn^{2+}$ ions are bound are the following:

$N^{\epsilon B29}$-hexanoyl des(B30) human insulin
$N^{\epsilon B29}$-octanoyl des(B30) human insulin
$N^{\epsilon B29}$-decanoyl des(B30) human insulin
$N^{\epsilon B29}$-dodecanoyl des(B30) human insulin
$N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-octanoyl $Gly^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-decanoyl $Gly^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ des(B30) human insulin

$N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-octanoyl $Ala^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-decanoyl $Ala^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ des(B30) human insulin
$N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-octanoyl $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-decanoyl $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ des(B30) human insulin
$N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ human insulin
$N^{\epsilon B29}$-octanoyl $Gly^{A21}$ human insulin
$N^{\epsilon B29}$-decanoyl $Gly^{A21}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ human insulin
$N^{\epsilon B29}$-octanoyl $Ala^{A21}$ human insulin
$N^{\epsilon B29}$-decanoyl $Ala^{A21}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-octanoyl $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-decanoyl $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Glu^{B30}$ human insulin

$N^{\epsilon B29}$-octanoyl $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-decanoyl $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-octanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-decanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin
$N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin

Examples of preferred human insulin derivatives according to the present invention in which two $Zn^{2+}$ ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-hexanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$

($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$

($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $2Zn^{2+}$

Examples of preferred human insulin derivatives according to the present invention in which three $Zn^{2+}$ ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-hexanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$

($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$

($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $3Zn^{2+}$

Examples of preferred human insulin derivatives according to the present invention in which four $Zn^{2+}$ ions are bound per insulin hexamer are the following:

($N^{\epsilon B29}$-hexanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$

($N^{\epsilon B29}$-dodecanoyl des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ des(B30) human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ human insulin)$_6$, $4Zn^{2+}$

($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gly^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Ala^{A21}$ $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-hexanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-octanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-decanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$
($N^{\epsilon B29}$-dodecanoyl $Gln^{B3}$ $Glu^{B30}$ human insulin)$_6$, $4Zn^{2+}$

## DETAILED DESCRIPTION OF THE INVENTION

### Terminology

The three letter and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. 243(1968), 3558.

In the DNA sequences A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms will be used:

DMSO for dimethyl sulfoxide.

DMF for dimethylformamide.

Boc for tert-butoxycarbonyl.

RP-HPLC for reversed phase high performance liquid chromatography.

X-ONSu represents an N-hydroxysuccinimid ester, X being an acyl group.

TFA for trifluoroacetic acid

### Preparation of hydrophobic insulin derivatives

The insulin moiety of the insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Derivatives featuring a residue in position B30, e.g. threonine (human insulin, HI) or alanine (porcine insulin, PI).

1.1 Starting from human insulin.

HI + Boc-reagent (e.g. di-tert-butyl dicarbonate) followed by HPLC purification

→ $Boc_2$-(A1,B1) HI

+ X-ONSu (where X represents the acyl moiety of a carboxylic acid)

→ X-(B29),$Boc_2$-(A1,B1) HI

+ TFA

→ X-(B29) HI

1.2 Starting from single chain precursors, extended in position B1. The extension (Ext) is connected to B1 via an arginine residue. The bridge from B30 to A1 is an arginine residue.

Ext-Arg-B(1-30)-Arg-A(1-21) precursor

+ X-ONSu

→ X-(B29),X-Ext-Arg-B(1-30)-Arg-A(1-21) precursor

+ trypsin in mixture of water and organic solvent

→ X-(B29),Arg(B31) HI

+ carboxypeptidase B

→ X-(B29) HI

2. Derivatives lacking a residue in position B30, i.e. des(B30) insulins.

2.1 Starting from HI or porcine insulin (PI)
PI/HI + carboxypeptidase A in ammonium buffer

→ des-(B30) insulin

+ Boc-reagent followed by HPLC purification

→ $Boc_2$(A1,B1) des(B30) insulin

+ X-ONSu (where X represents acyl moiety of fatty acid)

→ X-(B29),$Boc_2$(A1,B1) des-(B30) insulin

+ TFA

→ X-(B29) des-(B30) insulin

2.2 Starting from single chain precursors, extended in position B1. The extension (Ext) is connected to B1 via an

arginine residue. The bridge from B30 to A1 is a peptide $Y_n$-Arg, where Y is a codable amino acid except lysine and arginine, and n is an integer between 0 and 35.

Ext-Arg-B(1-30)-$Y_n$-Arg-A(1-21) precursor

+ lysyl endopeptidase, e.g. A lyticus protease

→ Ext-Arg-B(1-29),Thr-$Y_n$-Arg-A(1-21)des(B30) insulin

+ X-ONSu

→ X-(B29),X-Ext-Arg-B(1-29),X-Thr-$Y_n$-Arg-A(1-21)des-(B30) insulin

+ trypsin in mixture of water and organic solvent

→ X-(B29),des(B30) insulin

**Table 1. Data on $N^{\epsilon B29}$ modified insulins.**

Average of blood glucose values, in percent of initial level, after injection of 12 nmol of $N^{\epsilon B29}$-substituted insulin derivatives to rabbits, using 6 animals in the single day retardation test. IP is the scaled index of prolongation, see p. 211 in Markussen et al. Protein Engineering **vol. 1**, 205-213, 1987. It has been calculated from the glucose values. The formula has been scaled to render a value of 100 with bovine ultralente insulin and a value of 0 with Actrapid® insulin.

The derivatives have been administered in solutions containing 0 or 3 $Zn^{2+}$ ions per insulin hexamer.

The lipophilicity of an insulin derivative relative to human insulin, $k'_{rel}$, is measured on a LiChrosorb RP18 (5μm, 250x4 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water as eluents. The elution was monitored by following the UV absorption of the eluate at 214 nm. Void time $t_0$ is found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, $t_{human}$, is adjusted to at least $2t_0$ by varying the ratio between the A and B solutions. $k'_{rel} = (t_{derivative}-t_0)/(t_{human}-t_0)$.

| Derivative ~~Analogue~~ of human insulin, $Zn^{2+}$ ions/hexamer insulin | Blood glucose, % of initial | | | | IP | $k'_{rel}$ |
|---|---|---|---|---|---|---|
| | 1h | 2h | 4h | 6h | | |
| $N^{\epsilon B29}$-octanoyl human insulin, 3 $Zn^{2+}$ | 57.1 | 54.8 | 69.0 | 78.9 | 33 | |
| $N^{\epsilon B29}$-decanoyl human insulin, 0 $Zn^{2+}$ | 67.3 | 55.6 | 62.4 | 69.8 | 51 | 12.3 |
| $N^{\epsilon B29}$-decanoyl human insulin, 3 $Zn^{2+}$ | 73.3 | 59.4 | 64.9 | 68.0 | 60 | |
| $N^{\epsilon B29}$-myristoyl human insulin, 0 $Zn^{2+}$ | 99.6 | 92.4 | 91.0 | 93.7 | 78 | |
| $N^{\epsilon B29}$-decanoyl, des(B30) human insulin, 0 $Zn^{2+}$ | 74.9 | 67.0 | 67.1 | 69.8 | 61 | 11.0 |
| $N^{\epsilon B29}$-decanoyl, des(B30) human insulin, 3 $Zn^{2+}$ | 74.3 | 65.0 | 60.9 | 64.1 | 65 | |
| $N^{\epsilon B29}$-Boc human insulin, 0 $Zn^{2+}$ | 57.6 | 56.8 | 78.0 | 85.1 | 23 | 1.6 |
| $N^{\epsilon B29}$-phenylacetyl human insulin, 0 $Zn^{2+}$ | 55.4 | 58.9 | 88.8 | 90.1 | 10 | 1.3 |
| $N^{\epsilon B29}$-lithocholoyl human insulin, 0 $Zn^{2+}$ | 98.3 | 92.3 | 100.5 | 93.4 | 115 | 52 |
| $N^{\epsilon B29}$-deoxycholoyl human insulin, 0 $Zn^{2+}$ | 76.5 | 65.2 | 77.4 | 87.4 | 35 | 25 |

Table 1. Data on B29 modified insulins.

**Solubility.**

The solubility of all the above mentioned $N^{\epsilon B29}$ modified insulins exceeds 600 nmol/ml in a neutral (pH 7.5), aqueous, pharmaceutical formulation comprising 0.3% phenol as preservative, 3 $Zn^{2+}$ ions per insulin hexamer, and using either 1.6% glycerol or 0.7% sodium chloride to achieve isotonicity. 600 nmol/ml is the concentration of human insulin found in the 100 IU/ml compositions usually employed in the clinic.

The $\epsilon$-B29 amino group can be a component of an amide bond, a sulphonamide bond, a carbamide, a thiocarbamide, or a carbamate. The lipophilic substituent carried by the $\epsilon$-B29 amino group can also be an alkyl group.

Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

The injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure the human insulin derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride and glycerol.

Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

Compositions for nasal administration may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the activity of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

## EXAMPLES

**EXAMPLE 1**

Synthesis of A21 Ala, B3 Asp human insulin precursor from Yeast strain yEA002 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesised:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCT
CACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA
AGAGGTTTCTTCTACACTCCAAAGTCTGACGACGCT-3' (B3 Asp)

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTC
GTCAGACTTTGG-3' (A21 Ala)

#126 5'-GTCGCCATGGCTAAGAGATTCGTTG-3' (B3 Asp)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3'

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufactures instructions. In all cases were the PCR mix overlayed with 100 µl mineral oil (Sigma Chemical CO, St. Louis MO, USA)

2.5 µl oligonucleotide #98 (2.5 pmol)
2.5 µl oligonucleotide #128 (2.5 pmol)
10 µl 10X PCR buffer
16 µl dNTP mix
0.5 µl Taq enzyme
58.5 µl water

One cycle was performed: 94°C for 45 sec., 49°C for 1 min, 72°C for 2 min.

Subsequently, 5µl of oligonucleotides #16 and #126 was added and 15 cycles were performed: 94°C for 45 sec., 45°C for 1 min, 72°C for 1.5 min. The PCR mixture was loaded onto a 2.5 % aragose gel and electroforesed using standard techniques (Sambrook J, Fritsch El and Maniatis T, Molecular cloning, Cold Spring Harbour Laboratory press, 1989). The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean Kit (Bio 101 inc., PO BOX 2284, La Jolla, CA 92038, USA) according to the manufacturers instructions. The purified PCR DNA fragment was dissolved in 10 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and Xba I according to standard techniques, run on a 2.5% agarose gel and purified using the Gene Clean Kit as described.

The plasmid pAK188 consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader gene LaC212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor MI5 (for the DNA sequence, see Fig. 4) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK188 is shown in Fig. 1.

The plasmid pAK188 was also cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3139 bp isolated. The two DNA fragments were ligated together using T4 DNA ligase and standard conditions (Sambrook J, Fritsch EF and Maniatis T, Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). The ligation mix was transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using standard DNA miniprep technique (Sambrook J, Fritsch EL and Maniatis T, Molecular cloning, Cold Spring Harbour Laboratory Press, 1989), checked with appropriate restrictions endonucleases i.e. EcoRI, Xba I, NcoI, and HpaI. The selected plasmid was shown by DNA sequencing analyses (Sequenase, U.S.Biochemical Corp.) to contain the correct sequence for the A21 Ala, B3 Asp human insulin precursor and named pEA5.3.

The plasmid pKFN1627 consists of a DNA sequence of 520 bp encoding the yeast alpha leader followed by the insulin precursor MI5 (B1Glu, B28 Glu) (for the DNA sequence, see Fig. 5) inserted into the vector cpot. The vector pKFN1627 is shown in Fig. 1.

pEA5.3 was cut with the restriction endonucleases EcoRI and XbaI and the resulting the DNA fragment of 412 bp was isolated.

The yeast expression vector pKFN1627 was cut with the restriction endonucleases NcoI and XbaI and with NcoI and EcoRI and the DNA fragment of 9273 bp was isolated from the first digestion and the DNA fragment of 1644 bp was isolated from the second. The 412 bp EcoRI/XbaI fragment was then ligated to the two other fragments, that is the 9273 bp NcoI I/XbaI fragment and the 1644 bp NcoI/EcoRI fragment using standard techniques.

The ligation mix was transformed into E. coli as described above. Plasmid from the resulting E. coli was isolated using standard techniques , and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, Hpa I. The selected plasmid was shown by DNA sequence analysis (using the Sequenase kit as described by the manufacturer, US Biochemical) to contain the correct sequence for the A21 Ala, B3 Asp human insulin precursor DNA and to be inserted after the DNA encoding the LaC212spx3 signal/leader DNA. The plasmid was named pEA5.3.2 and is shown in Fig. 1. The DNA sequence encoding the LaC212spx3 signal/leader/A21 Ala, B3 Asp human insulin precursor complex is given in Fig. 6. The plasmid pEA5.3.2 was transformed into S. cerevisiae strain MT663 as described in European patent application 86306721.1 and the resulting strain was named yEA002.

**EXAMPLE 2**

Synthesis of A21 Ala, B3 Thr human insulin precursor from Yeast strain yEA005 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesised:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT
GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
CTCCAAAGTCTGACGACGCT-3' (B3 Thr)

#128 5'-CTGCGGGCTGCGTCTAAGCACAGTAGTTTTCCAATTGGTACAAA
GAACAGATAGAAGTACAACATTGTTCAACGATACCCTTAGCGTCG
TCAGACTTTGG-3' (A21 Ala)

#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (B3 Thr)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3'

The DNA encoding A21 Ala, B3 Thr human insulin precursor was constructed in the same manner as described for the DNA encoding A21 Ala, B3 Asp human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/A21 Ala, B3 Thr human insulin precursor complex is given in Fig. 7. The plasmid pEA8.1.1 was shown to contain the desired sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA005.

**EXAMPLE 3**

Synthesis of A21 Gly, B3 Asp human insulin precursor from Yeast strain yEA007 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesised:

#98 5'-TGGCTAAGAGATTCGTTGACCAACACTTGTGCGGTTCTCACTTG
GTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCT
ACACTCCAAAGTCTGACGACGCT-3' (B3 Asp)

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTC TCAGACTTTGG-3' (A21 Gly)

#126 5'-GTC ATGGCTAAGAGATTCGTTG-3' (B3 Asp)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3'

The DNA encoding A21 Gly, B3 Asp human insulin precursor was constructed in the same manner as described for the DNA encoding A21 Ala, B3 Asp human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/A21 Gly, B3 Asp human insulin precursor complex is given in Fig. 8. The plasmid pEA1.5.6 was shown to contain the desired sequence, transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA007.

**EXAMPLE 4**

Synthesis of A21 Gly, B3 Thr human insulin precursor from Yeast strain yEA006 using the LaC212spx3 signal/leader.

The following oligonucleotides were synthesised:

#101 5'-TGGCTAAGAGATTCGTTACTCAACACTTGTGCGGTTCTCACTT
GGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTCTTCTACA
CTCCAAAGTCTGACGACGCT-3' (B3 Thr)

#127 5'-CTGCGGGCTGCGTCTAACCACAGTAGTTTTCCAATTGGTACAA
AGAACAGATAGAAGTACAACATTGTTCAACGATACCCT
TAGCGTCGTCAGACTTTGG-3' (A21 Gly)

#15 5'-GTCGCCATGGCTAAGAGATTCGTTA-3' (B3 Thr)

#16 5'-CTGCTCTAGAGCCTGCGGGCTGCGTCT-3'

The DNA encoding A21 Gly, B3 Thr human insulin precursor was constructed in the same manner as described for the DNA encoding A21 Ala, B3 Asp human insulin precursor in Example 1. The DNA sequence encoding the LaC212spx3 signal/leader/A21 Gly, B3 Thr human insulin precursor complex is given in Fig. 9. The plasmid pEA4.4.11 was shown to contain the desired DNA sequence, transformed into S. cerevisiae strain MT663 as

described in Example 1 and the resulting strain was named yEA006.

**EXAMPLE 5**

Synthesis of B0 Arg, B31 Arg single chain human insulin precursor having an N-terminal extension (EEAEAEAR) from Yeast strain yEA113 using the alpha factor leader

A)

The following oligonucleotides were synthesised:

#220 5'-ACGTACGTTCTAGAGCCTGCGGGCTGC-3'

#263 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAAAGAGGTTTC TTCTACACTCCAAAGACTAGAGGTATCGTTGAA-3'

#307 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCTGAAGCT AGATTCGTTAACCAACAC-3'

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturers instructions. In all cases were the PCR mix overlayed with 100 µl of mineral oil (Sigma Chemical Co, St. Louis MO, USA).The plasmid pAK220 consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the insulin precursor MI5 (for the DNA sequence, see Fig. 4). inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA).

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #263 (100 pmol)

10 μl of 10X PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
0.5 μl of pAK220 plasmid as template (0.2 ug DNA)
63 μl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The PCR mixture was then loaded onto a 2% aragose gel and electroforesed using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 inc., PO BOX 2284, La Jolla , CA 92038, USA) according to the manufactures instructions. The purified PCR DNA fragment was dissolved in 10 μl of dest. water and restriction endonuclease buffer and cut with the restriction endonucleases HindIII and XbaI according to standard techniques. The HindIII/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK406 consists of a DNA sequence of 520 bp encoding the yeast alpha leader followed by the insulin precursor MI5 (for the DNA sequence, see Fig. 10) inserted into the vector cpot. The vector pAK406 is shown in Fig. 2.

The plasmid pAK233 consists of a DNA sequence of 412 bp encoding the synthetic yeast signal/leader LaC212spx3 (described in Example 3 of WO 89/02463) followed by the gene for the insulin precursor B(1-29) EKR A(1-21) (A21-Gly) (for the DNA sequence, see Fig. 11) inserted into the vector cPOT. The plasmid pAK233 is shown in Fig. 2.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 9273 bp isolated. The plasmid pAK406 was cut with the restriction endonucleases NcoI and HindIII and the vector fragment of 2012 bp isolated. These two DNA fragment was ligated together with the HindIII/XbaI PCR fragment using T4 DNA ligase and standard conditions . The ligation mix was then transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the B31Arg single chain human insulin precursor DNA and to be inserted after the DNA encoding the S. cerevisiae alpha factor DNA. The plasmid was named pEA108 and is shown in Fig. 2. The DNA sequence encoding the alpha factor leader/B31Arg single chain human insulin precursor complex are given in Fig. 12. The plasmid pEA 108 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA108.

B)

The following Polymerase Chain Reaction (PCR) was performed using the Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT 06859, USA) according to the manufacturers instructions. In all cases were the PCR mix overlayed with 100 µl of mineral oil (Sigma Chemical Co, St. Louis MO, USA)

5 µl of oligonucleotide #220 (100 pmol)
5 µl of oligonucleotide #307 (100 pmol)

10 μl of 10X PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
0.2 μl of pEA108 plasmid as template (0.1 ug DNA)
63 μl of water

A total of 16 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 40°C; and 2 minutes at 72°C. The PCR mixture was then loaded onto an 2% aragose gel and electroforesed using standard techniques. The resulting DNA fragment was cut out of the agarose gel and isolated using the Gene Clean kit (Bio 101 inc., PO BOX 2284, La Jolla , CA 92038, USA) according to the manufactures instructions. The purified PCR DNA fragment was dissolved in 10 μl of dest. water and restriction endonuclease buffer and cut with the restriction endonucleases NcoI and XbaI according to standard techniques. The NcoI/XbaI DNA fragment was purified using The Gene Clean Kit as described.

The plasmid pAK401 consists of a DNA sequence of 523 bp encoding the alpha leader (with a NcoI site added in the 3'-end) followed by the insulin precursor MI5 (for the DNA sequence, see 13 attached hereto) inserted into the vector (phagemid) pBLUESCRIPT IIsk(+/-) (Stratagene, USA). The plasmid pAK401 is shown in Fig. 3.

The plasmid pAK401 was cut with the restriction endonucleases NcoI and XbaI and the vector fragment of 3254 bp isolated and ligated together with the NcoI/XbaI PCR fragment. The ligation mix was then transformed into a competent E. coli strain and plasmids were isolated from the resulting E. coli colonies

using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI. The selected plasmid, named p113A (shown in Fig. 3), was cut with EcoRI and XbaI and the fragment of 535 bp isolated.

The plasmid pAK233 was cut with the restriction endonucleases NcoI and XbaI, and with EcoRI/NcoI and the fragments of 9273 and 1644 bp isolated. These two DNA fragment was ligated together with the EcoRI/XbaI fragment from p113A using T4 DNA ligase and standard conditions . The ligation mix was then transformed into a competent E. coli strain (R-, M+) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting E. coli colonies using a standard DNA miniprep technique and checked with appropriate restriction endonucleases i.e. EcoRI, XbaI, NcoI, HindIII. The selected plasmid was shown by DNA sequencing analyses to contain the correct sequence for the B31Arg single chain human insulin precursor DNA with the N-terminal extension EEAEAEAR and to be inserted after the DNA encoding the S. cerevisiae alpha factor DNA. The plasmid was named pEA113 and is shown in Fig. 3 attached hereto. The DNA sequence encoding the alpha factor leader/B0 Arg, B31 Arg single chain human insulin precursor having an N-terminal extension (EEAEAEAR) is given in Fig. 14 The plasmid pEA113 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA113.

**EXAMPLE 6**

Synthesis of B0 Arg, B31 Arg single chain human insulin precursor having an N-terminal extension (EEAEAEAER) from Yeast strain yEA136 using the alpha factor leader.

The following oligonucleotide was synthesised:

#389 5'-GCTAACGTCGCCATGGCTAAGAGAGAAGAAGCTGAAGCGAAG CTGAAAGATTCGTTAACCAACAC-3'

The following PCR was performed using the Gene Amp PCR reagent kit

5 μl of oligonucleotide #220 (100 pmol)
5 μl of oligonucleotide #389 (100 pmol)
10 μl of 10X PCR buffer
16 μl of dNTP mix
0.5 μl of Taq enzyme
2 μl of pEA113 plasmid as template (0.5 ug DNA)
63 μl of water

A total of 12 cycles were performed, each cycle comprising 1 minute at 95°C; 1 minute at 37°C; and 2 minutes at 72°C.

The DNA encoding alpha factor leader/B0 Arg, B31 Arg single chain human insulin precursor having an N-terminal extension (EEAEAEAER) was constructed in the same manner as described for the DNA encoding alpha factor leader/B0 Arg, B31 Arg single chain human insulin precursor having an N-terminal extension (EEAEAEAR) in Example 5. The plasmid was named pEA136. The DNA sequence encoding the alpha factor leader/B0 Arg, B31 Arg single chain human insulin precursor having an N-terminal

extension (EEAEAEAER) is given in Fig. 15. The plasmid pEA136 was transformed into S. cerevisiae strain MT663 as described in Example 1 and the resulting strain was named yEA136.

**EXAMPLE 7**

Synthesis of A1,B1-diBoc human insulin

5 g of zinc-free human insulin was dissolved in 41.3 ml of DMSO. To the solution was added 3.090 ml of acetic acid. The reaction was conducted at room temperature and initiated by addition of 565 mg of di-tert-butyl pyrocarbonate dissolved in 5.650 ml of DMSO. The reaction was allowed to proceed for 5½ hour and then stopped by addition of 250 µl of ethanolamin and subsequently precipitated by addition of 1500 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. A yield of 6.85 g material was obtained.

A1,B1-diBoc insulin was purified by RP-HPLC as follows: The crude product was dissolved in 100 ml of 25% ethanol in water, adjusted to pH 3.0 with HCl and applied to a column (5 cm diameter, 30 cm high) packed with octadecyldimethylsilyl-substituted silica particles (mean particle size 15 µm, pore size 100 Å) and equilibrated with elution buffer. The elution was performed with mixtures of ethanol and 1 mM HCl, 0.3 M KCl at a flow of 2 l/h. The insulin was eluted by increasing the ethanol content from 30% to 45%. The appropriate fraction was diluted to 20% ethanol and precipitated at pH 4.8. The precipitated material was isolated by centrifugation and dried in vacuum. Thus 1.701 g of A1,B1-diBoc insulin was obtained at a purity of 94.5%.

**EXAMPLE 8**

Synthesis of ($N^{\epsilon B29}$-benzoyl human insulin)$_6$, $3Zn^{2+}$

400 mg of A1,B1-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 µl of a mixture of N-methylmorpholine and DMSO (1/9, vol./vol.). The reaction was conducted at 15°C and initiated by addition of 14.6 mg of benzoic acid N-hydroxysuccinimide ester dissolved in 132 µl DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 343 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum.

$N^{\epsilon B29}$-benzoyl human insulin was purified by RP-HPLC as described in Example 7. 230 mg was obtained. Recrystallisation from 15% aqueous ethanol containing 6 mM $Zn^{2+}$ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 190 mg.

**EXAMPLE 9**

Synthesis of ($N^{\epsilon B29}$-lithocholyl human insulin)$_6$, $3Zn^{2+}$

400 mg of A1,B1-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 µl of a mixture of N-methylmorpholine and DMSO (1/9, vol./vol.). The reaction was

conducted at 15°C and initiated by addition of 31.94 mg of lithocholic acid N-hydroxysuccinimide ester dissolved in 300 µl of DMF. The reaction was stopped after 2 hours by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum and 331 mg of material was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield was 376 mg.

B29-lithocholyl insulin was purified by RP-HPLC as described in Example 7. A final yield of 67 mg was obtained at a purity of 94%. Recrystallisation from 15% aqueous ethanol containing 6 mM $Zn^{2+}$ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 49 mg.

**EXAMPLE 10**

Synthesis of ($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $3Zn^{2+}$

400 mg of A1,B1-diBoc human insulin was dissolved in 2 ml of DMSO. To the solution was added 748 µl of a mixture of N-methylmorpholine and DMSO (1/9, vol./vol.). The reaction was conducted at 15°C and initiated by addition of 18.0 mg of decanoic acid N-hydroxysuccinimide ester dissolved in 132 µl of DMSO. The reaction was stopped after 60 minutes and the product precipitated by addition of 100 ml of acetone. The precipitated material was isolated by centrifugation and dried in vacuum. 420 mg of intermediate product was collected.

The Boc protecting groups were eliminated by addition of 4 ml of TFA. The dissolved material was incubated for 30 minutes and the product was then precipitated by addition of 50 ml of acetone. The precipitate was isolated by centrifugation and dried in vacuum. The yield of crude product was 420 mg.

The crude product was purified by RP-HPLC as described in Example 7. A final yield of 254 mg of the title product was obtained. The purity was 96.1%. Recrystallisation from 15% aqueous ethanol containing 6 mM $Zn^{2+}$ and 50 mM citrate at pH 5.5 gave crystals of the title compound which were isolated by centrifugation and dried in vacuum. The yield was 217 mg.

**EXAMPLE 11**

Synthesis of des(B30) human insulin

Synthesis of des(B30) human insulin was carried out as described by Markussen (Methods in diabetes research, Vol. I, Laboratory methods, part B, 404-410. Ed: J. Larner and S. Phol, John Weiley & Sons, 1984). 5 g of human insulin was dissolved in 500 ml of water while the pH value of the solution was kept at 2.6 by addition of 0.5 M sulphuric acid. Subsequently, the insulin was salted out by addition of 100 g of ammonium sulphate and the precipitate was isolated by centrifugation. The pellet was dissolved in 800 ml of 0.1 M ammonium hydrogen carbonate and the pH value of the solution was adjusted to 8.4 with 1 M ammonia.

50 mg of bovine carboxypeptidase A was suspended in 25 ml of water and isolated by centrifugation. The crystals are were suspended in 25 ml of water and 1 M ammonia was added until a

clear solution was obtained at a final pH of 10. The carboxypeptidase solution was added to the insulin solution and the reaction was allowed to proceed for 24 hours. A few drops of toluene were added to act as preservative during the reaction.

After 24 hours the des(B30) human insulin was crystallized by successive addition of 80 g of sodium chloride while the solution was stirred. The pH value was then adjusted to 8.3 and the crystallisation was allowed to proceed for 20 hours with gentle stirring. The crystals were isolated on a 1.2 $\mu$m filter, washed with 250 ml of ice cold 2-propanol and finally dried in vacuum.

**EXAMPLE 12**

Synthesis of A1,B1-diBoc-des(B30) human insulin

The title compound was synthesized by a method similar to that described in Example 7. The crude product was precipitated by acetone and dried in vacuum. The A1,B1-diBoc-des(B30) human insulin was purified by RP-HPLC as described in Example 7.

**EXAMPLE 13**

Synthesis of $N^{\epsilon B29}$-decanoyl-des(B30) human insulin

400 mg of A1,B1-diBoc human insulin was used as starting material for the synthesis of $N^{\epsilon B29}$-decanoyl-des(B30) human insulin. The crude product was precipitated by acetone, dried in vacuum and deprotected using TFA. The resulting product was precipitated by acetone and dried in vacuum. $N^{\epsilon B29}$-decanoyl-

des(B30) human insulin was then purified by RP-HPLC as described in Example 10.

**EXAMPLE 14**

A pharmaceutical composition comprising 100 nmol/ml of ($N^{\epsilon B29}$-decanoyl des(B30) human insulin)$_6$, $2Zn^{2+}$ in solution

$N^{\epsilon B29}$-decanoyl des(B30) human insulin (1.2 μmol) was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 μl) and a solution containing 0.75% of phenol and 4% of glycerol (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

**EXAMPLE 15**

A pharmaceutical composition comprising 100 nmol/ml of ($N^{\epsilon B29}$-decanoyl human insulin)$_6$, $2Zn^{2+}$ in solution

0.2 μmol of the title compound was dissolved in water (0.8 ml) and the pH value was adjusted to 7.5 by addition of 0.2 M sodium hydroxide. 0.01 M zinc acetate (60 μl) and a solution containing 0.75% of phenol and 1.75% of sodium chloride (0.8 ml) was added. The pH value of the solution was adjusted to 7.5 using 0.2 M sodium hydroxide and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

**EXAMPLE 16**

A pharmaceutical composition comprising 100 nmol/ml of ($N^{\epsilon B29}$-litocholyl human insulin)$_6$, $2Zn^{2+}$ in solution

0.2 μmol of the title compound was suspended in water (0.8 ml) and dissolved by adjusting the pH value of the solution to 8.5 using 0.2 M sodium hydroxide. To the solution was then added 0.8 ml of a stock solution containing 0.75 % cresol and 4% glycerol in water. Finally, the pH value was again justed to 8.5 and the volume of the solution was adjusted to 2 ml with water.

The resulting solution was sterilized by filtration and transferred aseptically to a cartridge or a vial.

## CLAIMS

1. A protracted human insulin derivative, **characterized** in that the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg, and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg, and Cys; $Phe^{B1}$ may be deleted; the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent which comprises at least 6 carbon atoms; and 2-4 $Zn^{2+}$ ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and $Phe^{B1}$ is present then 2-4 $Zn^{2+}$ ions are bound to each hexamer of the insulin derivative.

2. An insulin derivative according to claim 1 wherein the B30 the amino acid residue is deleted.

3. An insulin derivative according to claim 1 wherein the B30 amino acid residue is Asp, Glu, or Thr.

4. An insulin derivative according to any one of the preceding claims wherein the A21 amino acid residue is Ala, Gln, Gly, or Ser.

5. An insulin derivative according to any one of the preceding claims wherein the B3 amino acid residue is Asp, Gln, or Thr.

6. An insulin derivative according to any one of the preceding claims, wherein the lipophilic substituent bound to the $\epsilon$-amino group of $Lys^{B29}$ is an acyl group derived from a

carboxylic acid having at least 6 carbon atoms.

7. An insulin derivative according to claim 6, wherein the acyl group, which may be branched, comprises a chain of carbon atoms 8 - 24 atoms long.

8. An insulin derivative according to claim 6, wherein the acyl group is the acyl group of a fatty acid having at least 6 carbon atoms.

9. An insulin derivative according to claim 6, wherein the acyl group is the acyl group of a linear, saturated carboxylic acid having from 6 to 24 carbon atoms.

10. Use of an insulin derivative according to any one of the claims 1 to 9 for the preparation of a medicament for treating diabetes.

11. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment comprising a therapeutically effective amount of an insulin derivative according to any one of the claims 1 to 9 together with a pharmaceutically acceptable carrier.

12. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment comprising a therapeutically effective amount of an insulin derivative according to any one of the claims 1 to 9, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

13. A method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to any one of the claims 1 to 9, together with a pharmaceutically acceptable carrier.

14. A method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to any one of the claims 1 to 9, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

**NOVO NORDISK A/S**

## ABSTRACT

# NOVEL PEPTIDES

A human insulin derivative in which the B30 amino acid residue is deleted or is any amino acid residue which can be coded for by the genetic code except Lys, Arg, and Cys; the A21 and the B3 amino acid residues are, independently, any amino acid residues which can be coded for by the genetic code except Lys, Arg, and Cys; $Phe^{B1}$ may be deleted; the $\epsilon$-amino group of $Lys^{B29}$ has a lipophilic substituent comprising at least 6 carbon atoms; and 2-4 $Zn^{2+}$ ions may be bound to each insulin hexamer with the proviso that when B30 is Thr or Ala and A21 and B3 are both Asn, and $Phe^{B1}$ is not deleted, then 2-4 $Zn^{2+}$ ions are bound to each hexamer of the insulin derivative.